



UNIVERSITI PUTRA MALAYSIA

**PRODUCTION, ESTABLISHMENT AND CHARACTERISATION
OF MONOCLONAL ANTIBODY AGAINST BREAST CANCER
CELL LINE (MCF-7)**

ONG BOO KEAN

FSMB 1995 2

**PRODUCTION, ESTABLISHMENT AND CHARACTERISATION
OF MONOCLONAL ANTIBODY
AGAINST BREAST CANCER CELL LINE (MCF-7)**

**By
ONG BOO KEAN**

**Thesis Submitted in Fulfilment of the Requirements
for the Degree of Master of Science in the
Faculty of Food Science and Biotechnology,
Universiti Pertanian Malaysia**

October 1995



Specially for.....

My respected parents,

Irene,

brothers B.Keang, B.Ping, B.Ching

ACKNOWLEDGEMENTS

I would like to express my most sincere appreciation to all the members of my supervisory committee; Dr. Abdul Manaf Ali, Prof. Madya Dr. Khatijah Mohd. Yusoff and Dr. Siti Aishah Mohd. Ali for their generous guidance, invaluable advice, support and encouragement which helped tremendously in the preparation of this thesis.

Appreciation is accorded to the Dean, Prof. Dr. Mohd. Mahyuddin Mohd. Dahan and to the Head of Department of Biotechnology, Prof. Dr. Mohd. Ismail Abdul Karim for the accessible of the facilities in the faculty throughout the course of this study.

I take the opportunity to record my thanks to all my friends and staff members of the Department of Biotechnology especially to Muhajir and En Rosli for their help throughout the course of the project. Deep appreciation is also accorded to all the staff members in the Histopathology Lab, UKM, especially En Zainal, Puan Kwan, En Aziz for their technical help during part of my study in UKM.

Last but not least, I would like to express my deepest gratitude to my beloved parents, brothers, sister in-law and Irene for their endless encouragement, patience and sacrifices which had helped me in my undertakings and to complete this research study.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF PLATES	xii
LIST OF ABBREVIATIONS	xiv
ABSTRACT	xv
ABSTRAK	xviii
 CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	5
Somatic Cell Hybridisation and Hybridoma Technology	5
Polyethylene Glycol as Fusogen	6
Mechanism of PEG-Induced Fusion	9
Cell Agglutination	10
Membrane Fusion	10
Cell Swelling	12
The Principles of Hybridoma Selection	13
Antibody Screening Assay	15
Cloning	18
The Properties and Significance of MAb as Compared to Polyclonal Antibody	19



	Improved Specificity	19
	Improved Sensitivity	20
	Unlimited Supply of Antibody	21
	Cost	21
	MAbs against Breast Cancer	22
	Human MAbs	26
	Biological Markers for Breast Cancer	27
	Epidermal Growth Factor	28
	Mammary Epithelial Membrane Antigen	28
	Oncogene and Oncoprotein	28
	Cytokeratin	29
	Carcinoembryogenic Antigen	30
	Cathepsin D	30
	Other Biological Markers	31
	MCF-7 Cell Line as an Immunogen	31
3	MATERIAL AND METHODS	33
	General Procedures	33
	Chemicals	33
	Cell Cultivation	33
	MCF-7 Cell Line	33
	Myeloma Cells	34
	Immunisation	34
	Cell ELISA Technique	35
	Fusion Procedure	36

Preparation of Myeloma Cells	36
Preparation of Lymphocyte Cells	37
Preparation of Feeder Cells	38
Fusion with Polyethylene Glycol (PEG)	38
Screening for Antibody-Producing Hybridomas	40
Limiting Dilution	40
Characterisation of Selected Clones and the Antigen Recognised by the MAb	41
Determination of Immunoglobulin Classes and Subclasses	41
Determination of Antigenic Determinant Specification by Cross-reactivities to Various Cell Lines	42
Analysis of Antigenic Determinant- Treatment with Biochemical Test	42
Immunofluorescence	43
Extraction of Antigen from MCF-7 Cells	44
Western Blotting	45
Polyacrylamide Gel Electrophoresis (PAGE)	45
Electroblotting	46
Dot Immunobinding Test	47
Immunohistochemistry	48
Tissue Sectioning Using Microtome	48
Immunostaining Using Labelled Streptavidin Biotinylated (LSAB) Method	48
Cultivation of Hybridoma Cells in Serum-free Medium	49



	Maintenance of C2E7 in Spinner Flask	50
	IgM Production of Hybridoma C2E7	51
	Partial Purification of MAb (IgM) Secreted from C2E7	52
4	RESULTS	54
	Production of Hybridoma Secreting Monoclonal Antibody (MAb) Against MCF-7	54
	Murine Antiserum Titre	54
	Cell Fusion Frequency and Efficiency of Producing Hybridoma	57
	Cell Cloning by Limiting Dilution	57
	Characterisation of Selected Hybridomas	62
	Determination of Antibody Classes and Subclasses	62
	Immunocytochemical Reactivity	66
	Analysis of Antigenic Determinant Using Biochemical Test	66
	Immunofluorescence	66
	Western Blotting and Dot Immunobinding	72
	Immunohistochemical Reactivity of MAb C2E7	72
	Maintenance of Clone C2E7 in Serum-Free Medium	79
	Maintenance of Clone C2E7 in Spinner Flask	79
	Partial Purification of MAb Secreted by Clone C2E7	86



5	DISCUSSION	92
	Production of Monoclonal Antibody (MAb) Against MCF-7 Cells	92
	Murine Polyclonal Antibody Titre	93
	Cell Fusion	93
	Single Cell Cloning by Limiting Dilution	96
	Characterisation of Selected Hybridoma Clones	99
	Determination of MAb Class and Subclass	99
	Analysis of Antigenic Determinant Using Biochemical Test	100
	Immunocytochemical Reactivity of MAb	101
	Immunofluorescence of MAb	103
	Western Blotting and Dot Immunoblotting	105
	Immunohistochemical Reactivity of MAb from Hybridoma C2E7	106
	MAb Against Breast Carcinoma	108
	Cultivation of C2E7 in Serum-Free Media	110
	Purification of MAb Secreted from Hybridoma Clone C2E7	112
6	SUMMARY	115
	BIBLIOGRAPHY	117
	APPENDICES	131
	VITA	146



LIST OF TABLES

Table	Page
1 The Percentage of Positive Wells of Various Hybridoma Clones at Different Limiting Dilution	58
2 Isotyping Classes and Subclasses for Selected Hybridoma Clones	65
3 Reactivity of MAb C2E7 to Various Human Cell Lines	67
4 Immunohistochemical Reactivity of MAb C2E7 against Various Human Tissue Sections	76
5 Proliferation of Murine Hybridoma C2E7 In Serum Free Defined Media	85
6 Partial Purification of MAb IgM from Cell Culture Supernatant of C2E7	88



LIST OF FIGURES

Figure	Page
1 The General Procedures of Monoclonal Antibody Production in Mice.	7
2 Mechanism of Cell-cell Fusion.	11
3 Metabolic Pathways Relevant to Hybrid Selection in Medium Containing Hypoxanthine, Aminopterin and Thymidine (HAT medium).	14
4 Solid-phase Binding Assay for Specific Antibody.	17
5 Value of Optical Density (O.D.) Values for Antiserum at Different Immunisation Week to Determine the Antiserum Titres	55
6 Murine Antiserum Titre at Different Weeks of Immunisation.	56
7 Percentage of Positive Well for Selected Hybridoma Clones After Each Limiting Dilution.	60
8 Number of Culture Plate Wells of C3A8 with Two to Five Times the O.D. Value of the Negative Control after Fifth Single-cell Cloning	61
9 Number of Culture Plate Wells of 10A2 with Two to Four Times the O.D. Value of the Negative Control after Fifth Single-cell Cloning	63
10 Number of Culture Plate Wells of C2E7 with Two to Thirteen Times the O.D. Value of the Negative Control after Fifth Single-cell Cloning	64
11 Reactivity of MAb C2E7 to MCF-7 Cell Line Treated with Trypsin.	68
12 Reactivity of MAb C2E7 to MCF-7 Cell Line Treated with Periodate.	69
13 Reactivity of MAb C2E7 to MCF-7 Cell Line Treated with Neuraminidase	70



14	Reactivity of MAb C2E7 against Different Fractions of MCF-7 Cells at varying Antigen Dilution	73
15	Growth Rate and Viability of C2E7 in Serum-Free and Serum Contained Media.	84
16	Viable Cell Growth and IgM Production of C2E7.	87
17	Protein Profile and Reactivity of Protein Fraction of Supernatant from C2E7 after Partial Purifying through Sepharose 6B.	89
18	Protein Standard Curve - Bradford Method.	143
19	IgM Standard Curve.	144



LIST OF PLATES

Plate	Page
1 Immunofluorescence Staining of MCF-7 Cells with MAb C2E7. Magnification 400 x.	71
2 Western Blotting Using MAb C2E7, After SDS-PAGE Separation of MCF-7 Homogenates, Followed by Electroblothing to Nitrocellulose Membrane.	74
3 A Dot Immunobinding Assay for MAb C2E7	75
4 Immunoperoxidase Staining of Human Invasive Ductal Breast Carcinoma. a) magnification 100 x, b) magnification 400 x.	77
5 Immunoperoxidase Staining of Human Invasive Lobular Breast Carcinoma. a) magnification 100 x, b) magnification 400 x.	78
6 Immunoperoxidase Staining of Human Breast Fibroadenoma. a) magnification 100 x, b) magnification 400 x.	80
7 Immunoperoxidase Staining of Human Malignant Tissue Sections. Magnification 100 x.	81
8 Immunoperoxidase Staining of Human Tissue Sections. Magnification 100 x.	82
9 Immunoperoxidase Staining of Human Breast Malignant Tumor with MAb C2E7. Heterogeneous Staining was Observed Within the Specimen in Certain Breast Cancer Tissues. Magnification 400 x.	83
10 Analysis of Purified IgM by Using 10% SDS-PAGE	91



11	MCF-7 Cell-Line was used as the Immunogen for Producing MAb against Breast Cancer.	130
12	Selection of Hybridoma Cells by Using Selective Media HAT.. . . .	131
13	The Development of Hybridoma Cells after Single-Cell Cloning Using Limiting Dilution Method	132



LIST OF ABBREVIATIONS

CGM	Complete Growth Medium
DMSO	Dimethyl Sulphoxide
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal Bovine Serum
HAT	Hypoxanthine, Aminopterin and Thymidine
HGPRT	Hypoxanthine-guanine-phosphoribosyl-transferase
HT	Hypoxanthine and Thymidine
ITES	Insulin, Transferin, Ethanolamine and Selenium
kDa	kiloDalton
MAb	Monoclonal Antibody
mg	milligram
ml	millimetre
MOPC	Mineral Oil Plasmacytoma
OD	Optical Density
PBS	Phosphate Buffer Saline
PEG	Polyethylene Glycol
RIA	Radioisotop Assay
RPMI	Rosewell Park Memorial Institute
TK	Thymidine-kinase
ul	microlitre
um	micrometer
%	percent
°C	degree Centigrade

Abstract of the thesis presented to the Senate of
Universiti Pertanian Malaysia in fulfilment of the requirements
for the degree of Master of Science.

**PRODUCTION, ESTABLISHMENT AND CHARACTERISATION
OF MONOCLONAL ANTIBODY
AGAINST BREAST CANCER CELL LINE (MCF-7)**

BY

ONG BOO KEAN

October, 1995

Chairman : Abdul Manaf bin Ali, Ph.D.

Faculty : Food Science and Biotechnology

Ever since hybridoma technology was introduced by Kohler and Milstein in the 70's, numerous efforts have been undertaken to produce monoclonal antibodies (MAbs) against mammary cancer cells. However, even to this day, all the MAbs produced still possess cross-reactivities toward other types of cancerous cells and also normal mammary cells.

In this study, the breast cancer cell line MCF-7 was used as an immunogen to raise MAb against mammary cancer cells. Fusion between lymphocytes sensitised with MCF-7 cell line and myeloma cells, SP2, was performed using 50% of polyethylene glycol (PEG). The hybridoma secreting MAb against MCF-7 cell line was selected using cell-ELISA technique. Limiting dilution of five times was performed to yield a stable hybridoma clone secreting the MAb.

The selected clone, C2E7, which secreted MAb of the IgM class and lamda light chains was chosen for further studies.

MAb secreted by C2E7 was found to react with an antigenic determinant located in the cytoplasm of the MCF-7 cell line. Immunocytochemical studies showed that apart from the MCF-7 cell line, the antigenic determinant was also present in mammary cancer cell line of T47-D. Weak cross-reactivities were also observed against cell lines Panc-1 and Ova-3. Immunohistochemical studies using the immunoperoxidase technique showed that staining occurred in the cytoplasmic region of all mammary lobular carcinoma and 90% of mammary ductal carcinoma examined. Staining was also found in 50% of mammary fibroadenoma cases studied. On the contrary, no staining of tissues was found in uterine leiomyoma, stomach showing intestinal metaplasia, cervical carcinoma, tonsillitis, neurofibroma, ductal papilloma of the breast and normal mammary tissues. Biochemical studies showed that the antigenic determinant on the MCF-7 cell line with reactivity towards MAb C2E7 was composed of endopeptide chain having arginine and lysine as the side chains, and possessed a specific conformational order which was disrupted when the determinant was electrophoresed on SDS-PAGE. Consequently, characterisation of the determinant using Western Blotting technique could not be performed.

The hybridoma clone C2E7 was able to grow and proliferate in serum-free medium of EDRF supplemented with ITES. Purification technique using a combination of ammonium sulphate precipitation and gel filtration on Sepharose 6B enabled the separation of IgM from MAb secretion of C2E7 hybridomas cultured in serum-free medium.

Abstrak tesis yang dikemukakan kepada Senat
Universiti Pertanian Malaysia sebagai memenuhi syarat
keperluan untuk Ijazah Master Sains

**PENGHASILAN, PEMBENTUKAN DAN PENCIRIAN
ANTIBODI MONOKLON
TERHADAP TITISAN SEL BARAH BUAH DADA MANUSIA (MCF-7)**

Oleh

ONG BOO KEAN

Oktober, 1995

Pengerusi : Abdul Manaf bin Ali, Ph.D.

Fakulti : Sains Makanan dan Bioteknologi

Semenjak teknologi hibridoma diperkenalkan Kohler dan Milstein pada tahun 70'an, pelbagai usaha telah dilakukan untuk menghasilkan antibodi monoklon (MAb) terhadap sel barah buah dada. Namun demikian, sehingga kini, hampir semua MAb yang telah dihasilkan itu masih mempunyai tindak-balas silang terhadap sel barah jenis lain dan juga sel normal buah dada.

Dalam kajian ini, titisan sel barah buah dada, MCF-7 telah digunakan sebagai immunogen dalam penghasilan Mab terhadap sel barah buah dada. Perlakuan antara sel limfosit yang telah diaruhkan dengan MCF-7 dan sel mieloma, SP2 dilakukan dengan 50% PEG. Teknik sel-ELISA telah digunakan untuk memilih hibridoma yang merembes antibodi terhadap titisan sel MCF-7. Pencairan terhad sebanyak lima kali dilakukan, agar klon

hibridoma yang stabil dalam rembesan MAb diperolehi. Seterusnya, hibridoma klon C2E7 yang merembes MAb kelas IgM dan rantai ringan lambda telah dipilih untuk kajian selanjutnya.

MAb dari C2E7 didapati bertindak dengan suatu penentu antigenik di sekitar sitoplasma titisan sel MCF-7. Kajian immunositokimia juga menunjukkan, selain di titisan sel MCF-7, penentu antigenik ini juga hadir di titisan sel barah buah dada T47-D. Tindak balas silang yang lemah juga didapati berlaku terhadap titisan sel Panc-1 dan Ova-3. Dalam kajian immunohistokimia dengan menggunakan teknik immunoperoxidase, didapati pewarnaan berlaku di kawasan sitoplasma pada semua tisu barah buah dada jenis karsinoma lobular dan 90% daripada tisu barah buah dada jenis karsinoma lobular yang telah diuji. Pewarnaan juga didapati pada 50% daripada kes fibroadenoma buah dada yang diuji. Sebaliknya, tiada sebarang pewarnaan berlaku pada tisu seperti uterine leiomyoma, karsinoma servik, tonsillitis, neurofibroma, perut menunjukkan intestinal metaplasia, duktal papilloma buah dada dan juga tisu normal buah dada. Ujian biokimia menunjukkan, penunjuk antigenik di titisan sel MCF-7 yang ditindak oleh MAb C2E7, terdiri dari rantai endopeptid dengan arginin dan lysin sebagai rantai sisi serta berada dalam keadaan konformasi spesifik. Konformasi spesifik ini akan berubah, andai kata ianya dielektroforeskan dalam SDS-PAGE. Oleh yang demikian, kaedah Western Blotting tidak dapat digunakan dalam pencirian penentu antigenik tersebut.

Hibridoma klon C2E7 berupaya hidup dan melakukan pembahagian sel dalam medium bebas serum, ERDF yang ditambah dengan ITES. Teknik penulenen yang melibatkan kombinasi pemendakan ammonium sulfat dan penurasan gel Sepharose 6B, membolehkan IgM, hasil rembesan hibridoma C2E7 yang dikultur dalam medium bebas serum, diasingkan.

CHAPTER 1

INTRODUCTION

Today, cancer is a major public health problem (Lim, 1991). Globally, out of the fifty million deaths that occur annually, five million is attributed to cancer. The World Health Organisation estimated that, by the year 2000, this figure will increase to eight million whereby 5 to 25% of this number is due to breast cancer (Management, 1994). The incidence of breast cancer is high in most of the industrialised and developed countries (Harris *et al.*, 1992). In the United States, breast cancer is the leading cause of death among women who are about forty to fifty-five years of age. The incidence rate of this disease has increased steadily, since formal tracking of such cases through cancer registries began in the 1930's. These incidences and mortality rates indicate that annually out of the 12% of American women diagnosed of having breast cancer, 3.5% of them will die of the disease (Harris *et al.*, 1992). In addition, according to Baum *et al.* (1991), about one in every 12 women in the United Kingdom will eventually develop this disease. Breast cancer is also one of the most common type of malignancy among women in Malaysia. About 1200 new cases of breast cancer are reported annually. Statistical data from the National Cancer Registry showed that cancer of the breast accounted for 10 and 18% of the total reported cancer and total female cancer cases respectively (Management, 1994).

Basically, death as a result of breast cancer is due to the distant spreading or metastasis of malignant tumour cells from the breast to the other vital organs of the body like the liver, lungs, bone and brain (Rosai, 1989). The progress of cancer can clinicopathologically be divided into four stages, namely, Stage I, II, III and IV (Chandrasoma and Taylor, 1991). Statistics showed that a patient suffering from Stage I of the disease and having a mass of less than 5 cm localised in the breast has 85% of the 5 year survival rate. However, patients with Stage IV of the disease where distant metastases have occurred, have only 10% of the 5 year survival rate (Chandrasoma and Taylor, 1991). Therefore, early detection of breast cancer is very important because the smaller the lesion, the greater is the likelihood of cure.

The detection and diagnosis of breast cancer is dependent upon the ability to discriminate between normal and neoplastic tissues. At present the histologic examination of a biopsy of a tumour mass is the most definitive diagnostic method of breast cancer (Chandrasoma and Taylor, 1991). For almost a century, routine histopathological diagnosis has been based upon the examination of haematoxylin and eosin stained paraffin embedded tissue sections. Although a majority of the tissues received in routine histopathology laboratories can be reliably diagnosed in this way, there are a number of cases whereby a firm diagnosis cannot be made on morphological grounds alone (Gatter *et al.*, 1982). To overcome such diagnostic problems diagnosis based on

immunohistological staining techniques were introduced. This approach although of genuine diagnostic value in some cases, has been limited in its scope by the relatively small number of tissue antigens which could be detected with conventional antisera. However, with the advent of hybridoma technology (Kohler and Milstein, 1975), the range of antigenic constituents which can be detected by immunohistological techniques in human tissue has been dramatically expanded (Gatter *et al*, 1982).

Several attempts to establish monoclonal antibodies (MAbs) specifically towards antigenic constituents in the breast cancer have been reported (Schlom *et al*, 1980; Taylow-Papadimitriou *et al*, 1981; Foster *et al*, 1982; Cordell *et al*, 1985; Pancino *et al*, 1989; Peterson *et al*, 1990; Pancino *et al*, 1991; Nuti *et al*, 1992; Modjtahedi *et al*, 1993). These MAbs differed in their binding capacities and their relative abilities to be sensitive and specific in recognising malignant cell lines as well as tissues of mammary origin. However, most of the reported MAb that react primarily with human breast carcinoma also showed reactivities towards other tumours and have considerable cross reactivities with normal human tissues. Consequently, to date, no MAb has yet been conclusively proven to be tumour specific for human breast carcinoma (Yuan *et al*, 1982; Plessers *et al*, 1990; Blottiere *et al*, 1991).

Thus, the objectives of this study are :

- a) to produce murine MAb against breast cancer cell line MCF-7;
- b) to characterise the selected hybridoma clones; and
- c) to cultivate a selected hybridoma clone in serum-free media and purify the MAb produced.